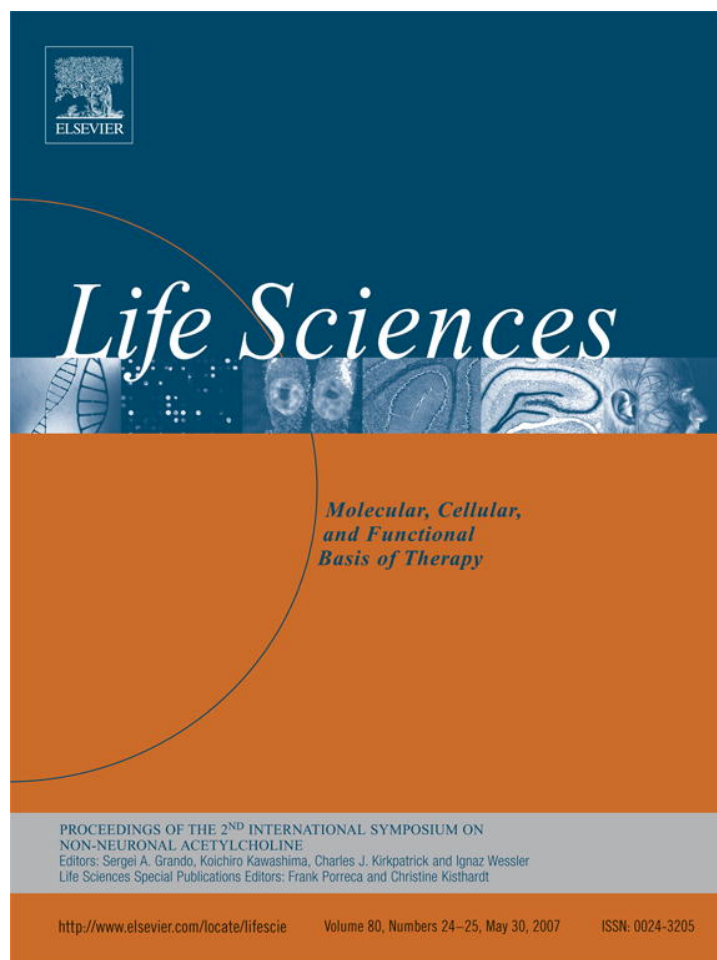


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C-reactive protein: A pentraxin with anti-acetylcholine activity

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Abstract

Purified C-reactive protein (CRP) diminished effects of acetylcholine (ACh) on the vascular tone and the heart rate of rats in vivo. In vitro CRP inhibited breakdown of ACh by acetylcholinesterase (AChE) while did not interact with AChE itself. CRP appears to bind ACh. CRP did not modify the cardiovascular effects of adenosine, another vasorelaxant. The data suggest that there is a new line of cross-talk between the inflammation and cholinergic regulation with CRP acting on endothelium via the ACh-dependent pathway.

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Introduction

Pentraxins represent a family of animal pentameric proteins binding phosphorylcholine (PC). Such pentraxins as C-reactive protein (CRP) and serum amyloid P component are the markers of acute phase of inflammation playing a protective role in innate immunity (Nazarov, 2001).

A similarity of PC to acetylcholine (ACh), a cell signalling molecule and parasympathetic neurotransmitter, has attracted our attention. To our knowledge ACh has not been ever mentioned among the ligands of CRP or other pentraxins, despite the fact that the CRP ligands such as lysophosphatidylcholine, sphingosine and lyso-platelet activating factor (all are the PC-containing compounds) were reported to exert the cholinergic effect and suppress the arterial relaxation response and the heart sensitivity to ACh (Prokazova et al., 1998).

People with cardiovascular events and low vascular response to ACh injection due to endothelial-vascular dysfunction show elevated blood levels of CRP (Fichtlscherer et al., 2004). Numerous data indicate that CRP participates in atherogenesis and arterial wall damage (Huang and Vita, 2006). This suggests that CRP can be implicated in the endothelial dysfunction.

The aim of the study was to investigate whether CRP could bind and inactivate ACh in vivo to limit its role in the regulation of the cardiovascular system. The following experiments were performed: 1) in vivo study on rats to elucidate CRP effects on the major ACh activities (hypotensive and heart slowing); 2) in vitro studies to test ACh binding by CRP.

Materials and methods

Animals and protocol of experiments

Male Wistar rats (Rappolovo Animal Farm, St. Petersburg, Russia), weighing 280–330 g (7 weeks old), were used. They were anaesthetized with i.p. sodium ethaminal, 50 mg/kg, dissolved in PBS. Two catheters were introduced: one into the femoral artery to record arterial pressure (AP) and another into the femoral vein for drug administration. After a 30-min stabilization period, responses were obtained to two hypotensive agents. Responses to ACh and adenosine (Ado) were recorded as percent fall in AP as compared to the individual animal's baseline AP before the response. The heart rate effects of the drugs were evaluated as a maximum change in R–R interval length on lead II electrocardiogram recorded at 50 mm/s using EK1T-03M2 recorder.

Each rat received two injections of a vasorelaxant with a 20-min interval. Group I rats received the 1st injection of

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ACh (2.5 µg per kg bodyweight in 0.1 ml of PBS) and, 20 min later, the 2nd injection of the same dose of ACh preincubated (15 min at room temperature) with either 0.1 ml of PBS (control) or 0.3 ml of CRP (650 µg/ml). Group II rats received the 2nd injection of ACh preincubated with human IgG (0.3 ml, 650 µg/ml) instead of CRP. Groups III and IV received two injections of Ado (2.5 µg/kg), which was mixed with PBS or CRP for the 2nd injection. The rats received approximately 4.6 nM of ACh and 2 nM CRP (CRP to ACh molar ratio was approximately 1:2.5, which corresponded to at least a 2-fold molar excess of PC-binding sites of CRP over ACh).

Hydrolysis of ACh by the method of Hestrin

Hydroxylamine reaction of the Hestrin method (Kao and Tsai, 2004) was used to estimate the ACh degradation by AChE in the presence of CRP or IgG. Readings were done at 570 nm with Spektra-III microplate reader (Austria).

Agarose gel electrophoresis

For agarose gel electrophoresis (without SDS) CRP, ACh, PC, pneumococcal C-polysaccharide (CPS) and AChE were dissolved

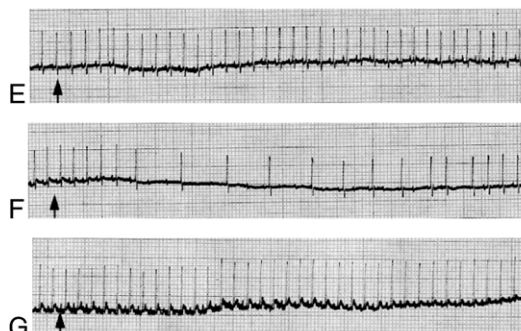
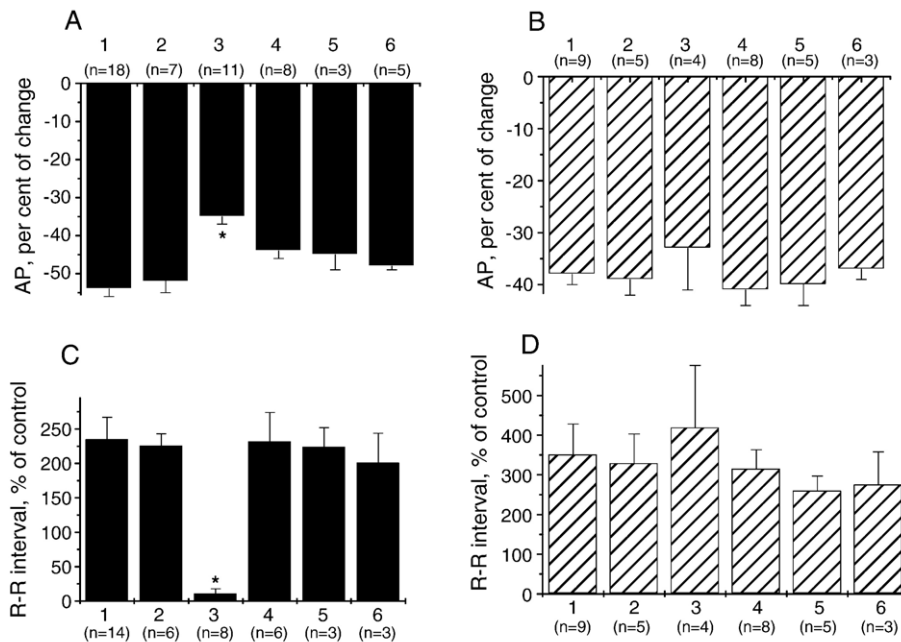
in Hanks balanced salt solution containing calcium ions since PC-binding sites of CRP are known to be calcium-dependent.

Drugs and reagents

ACh hydrochloride was purchased from Kazan Pharmaceuticals (Russia), purified human CRP from ICN Biomedicals (USA) and RDI (USA), Ado from ICN Biomedicals (USA), bovine erythrocytes' AChE (EC 3.1.1.7; 65 U/mg protein from Perm Institute of Epidemiology and Microbiology (Russia), normal human gamma-globulin (IgG) from Pasteur Institute (St. Petersburg, Russia), phosphorylcholine chloride (PC) from Sigma Chem. Co. (USA), pneumococcal C-polysaccharide (CPS) from Reanal (Hungary), reagents for Hestrin method and agarose gel electrophoresis from ICN Biomedicals, Helicon, and Vekton (St. Petersburg).

Statistics

The values are expressed as mean ± SEM with the number of animals or experiments (n) shown in brackets. The data was analyzed for statistical significance using Student's unpaired two-tailed t-test. The significance was considered at p value less than 0.05.



Results

CRP inhibits the responses to ACh *in vivo*

The i.v. administration of ACh caused a 50% decrease in mean AP compared to the background level (Fig. 1A). In parallel, a short-term slowing of the heart rate occurred: R–R interval became 2.5 times longer 5 s after ACh injection. Repeated injection of ACh after a 20-min rest period resulted in AP and R–R interval changes of the same character and magnitude as upon the first injection.

The administration of ACh premixed and preincubated with CRP resulted in a marked attenuation (by 33%, $p < 0.05$) of hypotensive effect of ACh. The rats which received ACh plus CRP demonstrated significantly less fall in the AP than the rats given with ACh plus PBS ($p < 0.05$) or the control animals which received ACh mixed with another protein (IgG) (Fig. 1A). The hypotensive response to ACh in the IgG+ACh treated rats was quite similar to the control group given with ACh plus PBS (Fig. 1).

Different results were obtained with Ado as a vasodilator (Fig. 1B). Ado preincubated with CRP did not moderate the AP

Fig. 1. Arterial pressure and heart rate changes in rats injected with a mixture of vasodilator (ACh or adenosine) and human C-reactive protein or IgG. A, C: ACh; B, D: Ado. Abscissa. 1, 2, 3: experiments with CRP; 4, 5, 6: experiments with IgG. 1, 4: 1st injection (ACh or Ado+PBS); 2, 5: 2nd injection (ACh or Ado+PBS); 3, 6: 2nd injection (ACh or Ado+CRP or IgG). The numbers of animals are indicated in parenthesis. Ordinate: change in arterial pressure or R–R interval length, % of control. Asterisk: significantly different from 1 and 2 ($p < 0.05$ or less). E, F, G, examples of electrocardiogram: E — after PBS, F — after ACh+PBS, G — after a mixture of ACh+CRP. Arrows point to the infusion.

Numerical data to Fig. 1A (acetylcholine)

Exp. set	Order of injections	Drugs injected	<i>n</i>	% change from baseline
1	1st injection	ACh+PBS	18	-54±2
2	2nd injection	ACh+PBS	7	-52±3
3	2nd injection	ACh+CRP	11	-35±2*
1	1st injection	ACh+PBS	8	-44±2
2	2nd injection	ACh+PBS	3	-45±4
3	2nd injection	ACh+IgG	5	-48±1

Student's *t* value=4.7; $p < 0.05$.

Numerical data to Fig. 1B (adenosine)

Exp. set	Order of injections	Drugs injected	<i>n</i>	% change from baseline
1	1st injection	Ado+PBS	9	-38±2
2	2nd injection	Ado+PBS	5	-39±3
3	2nd injection	Ado+CRP	4	-33±8
1	1st injection	Ado+PBS	8	-41±3
2	2nd injection	Ado+PBS	5	-40±4
3	2nd injection	Ado+IgG	3	-37±2

Numerical data to Fig. 1C (acetylcholine)

Exp. set	Order of injections	Drugs injected	RR interval	
			<i>n</i>	% change from control
1	1st injection	ACh+PBS	14	235±32
2	2nd injection	ACh+PBS	6	226±17
3	2nd injection	ACh+CRP	8	11±7*
1	1st injection	ACh+PBS	6	232±42
2	2nd injection	ACh+PBS	3	224±28
3	2nd injection	ACh+IgG	3	201±43

Student's *t* value=18.7; $p < 0.001$.

Numerical data to Fig. 1D (adenosine)

Exp. set	Order of injections	Drugs injected	RR interval	
			<i>n</i>	% change from control
1	1st injection	Ado+PBS	9	351±77
2	2nd injection	Ado+PBS	5	329±74
3	2nd injection	Ado+CRP	4	419±157
1	1st injection	Ado+PBS	8	315±48
2	2nd injection	Ado+PBS	5	260±37
3	2nd injection	Ado+IgG	3	275±83

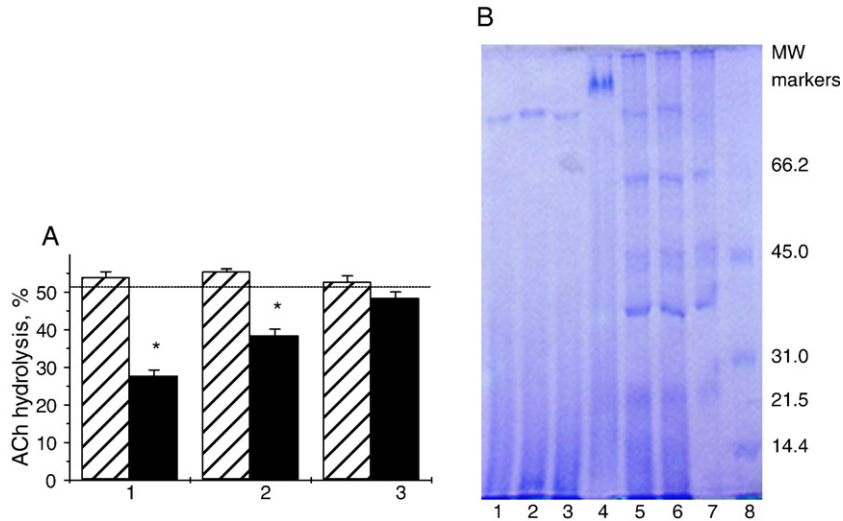


Fig. 2. Interactions of C-reactive protein with ACh and AChE. A: Inhibition of AChE-induced hydrolysis of ACh by CRP. Abscissa. Concentration of CRP (and human IgG): 1, 1.5 mg/ml, 2, 0.75 mg/ml, 3, 0.3 mg/ml. Hatched columns — IgG, filled columns — CRP. AChE was used at concentration of 1 mg/ml (approx. 65 U/ml). Ordinate. Percent of ACh hydrolysis. Given is the amount of ACh hydrolyzed after 30 min incubation with AChE in relation to that added at time zero (0.4 mg/ml ACh). One milligram of AChE was added to the mixtures. Dashed horizontal line ($51.4 \pm 2.8\%$) shows background level of ACh hydrolysis by AChE in the presence of PBS instead of CRP or IgG. Data of four experiments are expressed as the mean values \pm SEM. Asterisk — significantly differed ($p < 0.05$) from correspondent IgG column. B: Electrophoretic mobility of CRP and its mixtures with ACh and AChE. Electrophoresis in agarose gel. Lanes: 1, CRP+ACh; 2, 3, CRP; 4, CRP+pneumococcal C-polysaccharide; 5, 6, CRP+AChE; 7, AChE; 8, molecular weight markers.

Numerical data to Fig. 2A

	IgG		CRP	
	Mean	Standard error of mean	Mean	Standard error of mean
1	53.95	1.45	27.7	1.6
2	55.4	0.7	38.4	1.7
3	52.7	1.65227	48.35	1.68201

fall compared to the effect of control Ado+PBS combination ($p > 0.05$). The effect of Ado+IgG mixture did not differ from the effect of Ado+PBS control mixture (Fig. 1B, $p > 0.05$).

Cardiac effects of ACh and Ado and their susceptibility to the modulation with CRP and IgG are presented on Fig. 1C, D. Both agents induced significant lengthening of R–R interval. CRP premixed and injected with ACh exerted profound effect on R–R interval compared to ACh+PBS (Fig. 1D, $p < 0.05$). CRP almost completely abolished the ACh effect on the heart rate.

In contrast, the results with Ado were quite different. The increase in the R–R interval induced by Ado was insensitive to CRP (Fig. 1D), and the difference between Ado+CRP and Ado+PBS groups was not significant ($p > 0.05$).

Fig. 1E–F represents samples of electrocardiograms recorded in the control rats and in the rats injected with ACh+CRP. The difference between ACh effects in the absence and the presence of CRP is obvious.

CRP inhibits ACh hydrolysis by AChE in vitro

Fig. 2A shows the effects of human CRP and IgG on hydrolysis of ACh by AChE in vitro. CRP inhibited ACh breakdown by AChE in a dose-dependent way (Fig. 2A, $p < 0.05$). Unlike CRP, IgG did not inhibit ACh degradation

(Fig. 2A). These data suggest that CRP may bind and protect ACh from the enzymatic destruction.

CRP does not interact with AChE

The mixture of CRP with ACh displayed the same electrophoretic migration pattern as CRP alone (Fig. 2B). Low molecular weight ACh did not change the charge and mobility of CRP. The comparison of lanes 5 and 6 (CRP mixed with AChE on both) with the lane 7 (AChE alone) shows that CRP does not interact with the enzyme. The lane 4 provides an example of typical interaction of CRP with its classical PC-containing ligand, pneumococcal C-polysaccharide. Thus, the inhibitory effect of CRP on ACh breakdown by AChE was most likely a result of the ACh capture rather than the action on the enzyme.

Discussion

Our study shows for the first time the relationship between CRP, the best characterized member of pentraxin family, and neurotransmitter ACh, an important parasympathetic mediator and emerging non-neuronal factor of the lymphoid system (Kawashima and Fujii, 2000; Wessler and Kirkpatrick, 2001; Kirkpatrick et al., 2003; Wessler et al., 2003). The vasodilatory effect of ACh is known to be mediated by NO pathway (Han

et al., 1998). Inflammation down-regulates eNOS activity and also reduces the bioavailability of NO (Huang and Vita, 2006). The interactions of CRP with the NO generation in endothelium are complex and poorly elucidated. CRP was shown both to down-regulate the eNOS expression (Mineo et al., 2005) and, on the contrary, to protect the eNOS protein expression from the suppression by TNF- α (Escribano-Burgos et al., 2005). According to our data, the inhibitory effect of CRP on the cardiovascular response to ACh is unlikely due to the interference with the NO generation or action, since CRP had no effect on the vasodilatory effect of ADo, a vasorelaxant, whose mechanism of action includes NO as well but does not involve ACh receptors. Evidently the inhibitory effect of CRP on ACh-induced vasorelaxation in our experiments was mediated through the ACh-dependent pathway.

CRP might act on ACh itself (by capturing it) or on cellular ACh receptors (by blocking them). The results showed that CRP did not interact with AChE but nevertheless inhibited ACh hydrolysis by this enzyme. This fact provides a considerable evidence for the ACh-capturing mechanism. Electrophoresis of the CRP+ACh mixture might not be a proper way for testing such a question, because of a much smaller size of the ACh molecules compared to CRP (167 vs 107,000 Da) and their negligible influence on the charge and electrophoretic mobility of CRP. However, the electrophoresis data are quite suitable to exclude interaction between large molecules of CRP and AChE. A possibility of blocking the ACh receptors by CRP needs to be tested further.

The in vivo and in vitro results correlate well. The ability of CRP to bind ACh fits the results obtained in vitro with AChE and in vivo on the rats. ACh molecules captured by CRP should be sterically hidden and inaccessible both for a hydrolytic enzyme or cell membrane receptors.

CRP levels in normal blood are low but increase during inflammation. So the effects of CRP should be limited by the acute phase of inflammation. However, as had been shown earlier, CRP can be produced not only in the liver during the systemic inflammation, but also in lymphoid cells upon their activation (Nazarov and Sofronov, 1983; Ikuta et al., 1986). Therefore, locally synthesized CRP might participate in the processes regulating by non-neuronal ACh. The role of the CRP–ACh interactions in the regulation of the defense reactions has to be further evaluated.

Acknowledgements

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